UNIVERSITY OF PENNSYLVANIA

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# MEMBRANES IN THE PLANT CELL

JANET QUENTIN PLOWE

L. C.

A THESIS

IN BOTANY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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# MEMBRANES IN THE PLANT CELL

## I. MORPHOLOGICAL MEMBRANES AT PROTOPLASMIC SURFACES

JANET Q. PLOWE

With 11 Text-figures

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## INTRODUCTION

The question of the existence and function of plasma membranes is of concern to the physiologist both from a practical and from a theoretical point of view. It is of practical interest because, in attempting to analyze the action of chemicals on the living organism, it is essential to know whether substances in the external medium necessarily come into direct contact with all internal constituents of the cell which they affect, or whether action on an external regulatory layer alone may be responsible for changes in other parts of the cell which the chemicals never reach. It is of theoretical interest because, if it can be shown that the existence and function of plasma membranes is essential to the life of the protoplast, it follows that it is as incorrect to think of protoplasm as a living substance apart from its organization into protoplasts as it would be to think of proteins, or water, or carbohydrates as living unless organized into protoplasm.

In that case, the protoplast, rather than protoplasm, becomes "the physical basis of life".

The concept of specialized, differentially permeable layers in the cell is so convenient in the explanation of cell phenomena that it has widespread acceptance. The statement is made in a current text on plant physiology, with no implication of uncertainty, that "The nucleus, plastids, etc., as well as the cytoplasm, have surface membranes on the inner and outer surfaces which permit certain substances to pass through while

keeping others from doing so. The cell is able to carry on many different processes at the same time, owing to these various membranes which surround the different structures in it" (RABER 1928, p. 13).

The view that membranes surround nucleus, vacuole, and protoplast is generally accepted by cytologists (SHARP 1926, p. 57; WILSON 1925, pp. 54—55, 85). Yet the membrane theory is not only disputed by those who propose, instead, an explanation of self-regulatory ability based on the behavior of a colloidal system rather than on a complex cell structure, but it is granted by its adherents that the experimental evidence on which it rests is inadequate. HÖBER, who has contributed both experimental work and theoretical consideration in support of the theory, states that „Einen einwandfreien direkten Nachweis der Plasmahaut gibt es danach bisher überhaupt nicht" (1926).

The question calls for further investigation, not only on its own merits, but because an answer is of importance in physiological work along other lines.

## PREVIOUS INVESTIGATIONS

In the last century, DE VRIES (1885) isolated the layer of cytoplasm surrounding the plant vacuole by rapid plasmolysis. He made a study of the permeability and physical properties of this layer, which he called the tonoplast. He appears to have looked upon the isolated tonoplast as living, since it might divide, but probably the division he saw resulted from surface tension forces alone. More significant, perhaps, is the fact that the differential permeability of the tonoplast so closely parallels that of the entire living protoplast that it seems unlikely that the tonoplast originates as an artifact.

PFEFFER (1897) demonstrated that the outer surface of a plant protoplast retained differential permeability for dyes when killed with acid, although, when the surface layer was ruptured, the dyes penetrated and stained the cytoplasm.

In view of our present knowledge of the radical changes in physical properties undergone by dying protoplasm, both these pioneer contributions to the physiological morphology of the cell must be considered an uncertain basis for conclusions regarding plasma membranes in the living protoplast.

Comparatively little of the recent investigation of physical properties of protoplasm has been directed toward the question of differentiation of surface layers. Microscopic observation ordinarily fails to reveal such a layer at either cytoplasm-wall or cytoplasm-sap interface, in the plasmolyzed or unplasmolyzed cell; however PRICE (1914) using dark field illumination, was able to detect the presence of an outer layer distinct from the remainder of the plasmolyzed *Spirogyra*, *Mougeotia*, or *Cucurbita* protoplast. In the first, the layer contains fine particles in rapid motion; the second shows no movement, suggesting that it is in the gel condition; in the third, the layer is homogeneous. In *Spirogyra* a similar layer surrounds the vacuole. PRICE also notes that the strands which persist between plasmolyzed protoplast and wall in *Cucurbita* are like the material composing the outer layer, from which their origin is clearly discernible in some cases.

KÜSTER (1910) describes the appearance of a fine membrane in cells of *Allium* treated with acid on death from rapid deplasmolysis; this later collapses against the plasma mass. He reports (1926) that when a protoplast divides on plasmolysis, a thin strand of tonoplast may persist, running through the strand of cytoplasm connecting the two portions of the protoplast. The strand of tonoplast becomes evident on "foamy degeneration" of the cytoplasm. In another paper (1927) he describes the occurence of naked vacuolar membranes in the sap of ripe solanaceous berries, where they could hardly be considered to result from the use of reagents.

GICKLHORN and WEBER (1927) report that when mesophyll cells are placed in conductivity water or in isotonic solutions, the vacuole, surrounded by a thin layer of protoplasm, may contract, although the remainder of the protoplasm remains next to the cell wall. When these cells are placed in stronger solutions, the protoplast undergoes normal plasmolysis and the vacuole contracts further. The thin layer about the vacuole may be a distinct osmotic membrane.

On the basis of measurable potential differences between vacuolar sap and the external artificial sap in which cells of *Valonia* are immersed, OSTERHOUT, DAMON, and JACQUES (1927) conclude that the inner and outer layers of the protoplast differ.

## THE MICROMANIPULATION METHOD

Some time ago PFEFFER (1897) remarked that the question of the presence of differentially permeable membranes "Would be definitely answered . . . if it were found that substances commonly present or artificially introduced, diffused through the central mass of the plasma, but did not appear in the vacuole or in the water outside." PFEFFER's idea can now be carried out, using fine glass pipettes and needles in connection with a micromanipulation device.

The original term, "microdissection", should be abandoned, for dissection implies dismemberment of a dead organism, and micromanipulative methods are now associated with the study of the internal physiology of the living cell. It is by such means that the internal conductivity, the internal hydrogen ion concentration, and the internal oxidation-reduction potential of active, living cells are now being investigated.

Less work with micromanipulation apparatus has been done on plant cells than on animal cells, because the layer of protoplasm in the plant cell is relatively thin and the cell wall offers an obstacle to the insertion and free movement of needles or pipettes.

KITE (1913 a, b; 1915) who was the first to publish extensive reports of micromanipulation experiments, attempted to investigate the consistency of cytoplasm and nucleus and the relative permeability of the internal and external cytoplasmic layers in plant as well as in animal cells. It is unfortunate that in many instances he made no distinction between dead and living protoplasm. This lack of discrimination causes uncertainty in interpreting his accounts.

SEIFRIZ (1921) employed such plant material as *Vaucheria*, pollen tubes, and bread mould in investigations of the physical properties of protoplasm, working chiefly on expressed cytoplasm.

CHAMBERS and SANDS (1923) used the pollen mother cells of *Tradescantia*, in some cases piercing the cell wall and in older cells removing the wall before dissection. They directed their attention towards mitotic structures. Here again the distinction between dead and living structure was not always carefully drawn.

Scarth (1927) contributed the first account of investigation of the interior of a plant cell indisputably alive and in normal condition. His material included *Tradescantia*, *Elodea*, *Symphoricarpos*, and *Spirogyra*. He used needles with horizontal tips, piercing the end walls of the cells. Lateral and vertical movement were made possible by the extreme flexibility of the needles. Scarth was chiefly interested in the internal structural organization of cytoplasm and nucleus.

Seifriz and Höfler (1927) subsequently described a method by which the interference of the cell wall could be done away with and living, plasmolyzed protoplasts reached with the needle as freely as in animal cells. This method, to be described later, gives admirable material for investigation of the protoplast. Not only is the needle or pipette free to move in any direction, but the outer layer of the cytoplasm is not in contact with the cell wall.

## IS A CELL UNDERGOING MICROMANIPULATION A NORMAL CELL?

It is justifiable to ask whether the behavior of a protoplast into which microneedles or micropipettes are inserted can give us any information about the nature of the protoplast under normal conditions. Those who have opportunity to watch the process of micromanipulation can hardly fail to be reassured by the astounding indifference of the protoplast to the insertion and movement of needles and to injection. Others may be enabled to judge to what extent protoplasts subjected to micromanipulation retain normal behavior by consideration of the following illustrations.

Taylor and Whitaker (1927) find that streaming may continue in *Nitella* for more than ten days after microelectrodes are inserted into the protoplast. Taylor and Farber (1924) investigating the function of the micronucleus in *Euplotes*, show that, after the micronucleus has been removed with a micropipette, the organism continues its existence as an individual, but fails to divide. The micronucleus can, however, be removed and immediately reinjected without interfering with reproduction.

Seifriz (1929 a) finds that an injected nickel particle may be carried along in streaming myxomycete protoplasm. Chambers, Pollack, and Hiller (1927) state that *Amoeba proteus* and *Amoeba dubia* injected with phenol red may retain normal behavior for forty-eight hours. Pollack (1928) finds that amoebae injected with alizarin sulfonate recover normal movement more quickly if calcium salts are injected.

Such instances are evidence that interference with normal function does not necessarily follow the insertion of microneedles into the living protoplast, or the injection into it of material from a micropipette.

## LOCAL, REVERSIBLE INJURY IN THE PROTOPLAST

Protoplasts, like organisms, may be subject to reversible injury. Temporary changes in properties accompanying reversible injury have been made familiar by the work of Osterhout (1922). A second question therefore arises: Is it not likely that the movement of microneedles or the force of an injection produces local alterations in the protoplast and renders results inaccurate, even though death of the protoplast does not follow? The possibility of such transitory local injury changes within the protoplast is suggested by workers with micromanipulation. Survival of the cell is not a satisfactory

guarantee, for them, that the behavior of the protoplast has been normal throughout the investigation. Such signs as cessation or increase of Brownian movement, aggregation of granules, swelling, must constantly be watched for. The appearance of acidity may be used in detecting local injury. CHAMBERS and POLLACK (1927) and CHAMBERS, POLLACK, and HILLER (1927) have shown that the reaction of the protoplasm of certain animal cells changes from its normal level (pH 6.7—7.5) to as low as pH 5.3 on fatal injury. REZNIKOFF and POLLACK (1929) state that, although a churning motion with a needle or pipette causes a distinct temporary local acidity in the cytoplasm of *Amoeba dubia*, the ordinary quiet insertion of a needle, or even the injection of NaCl or KCl solution, causes no change in reaction.

## PROTOPLASMIC STREAMING IN PLANT CELLS AS A CHECK ON NORMAL CONDITION

Changes in protoplasmic streaming offer, in many plant cells, a sensitive indicator of changes in the condition of the protoplasm. While protoplasm in cells which normally show streaming may cease streaming without having undergone appreciable injury, it is also true that known injury is regularly accompanied by changes in the manner of streaming or by complete cessation. If we disregard all results in which streaming is affected, we may feel reasonably sure that the behavior of the protoplasm in other respects will not be abnormal.

SCARTH has reported that a strand of cytoplasm traversing the vacuole may continue streaming while stretched by a microneedle. TAYLOR's experience with *Nitella* and SEIFRIZ' with mycomycetes were cited above. To these may be added the following facts from the present work. When a needle is pushed into the protoplast of an onion cell, carrying a layer of cytoplasm with it, streaming will take place in the layer about the needle in a wholly normal manner. Strands may form running from the needle to the wall. The nucleus may move out along the needle to its tip. Further, if the needle is thrust through the protoplast until it touches the protoplast-vacuolar surface on the opposite side, then partially withdrawn, a strand of protoplasm will be pulled out from that surface into the vacuole, and in this strand, too, normal streaming will occur. Or, if the needle is withdrawn from the plasmolyzed protoplast, pulling a strand of protoplasm out after it, streaming will occur in this strand both towards and away from the protoplast. A protoplast may be pinched in two with the needle, and yet streaming in the two portions continue undisturbed. An opening may be made through the protoplast which brings the vacuole into communication with the external solution, and yet streaming continue uninterrupted.

These results encourage us to believe that, with careful manipulation, it is possible to carry on intracellular investigations of plant protoplasts as well as of animal protoplasts in which the protoplasm under observation is normal insofar as any organism, organ, or tissue can be considered normal while under experimental conditions.

## MICROMANIPULATIVE STUDIES OF THE PRESENCE OF MEMBRANES

Evidence from microdissection is generally in support of the presence of differentiated layers at protoplasmic surfaces. CHAMBERS (1917) states that *Paramoecia* and marine over can recover from dissection only if a new film, or series of films, is formed, cutting off the injured region.

Chambers and Rényi (1925) report that tearing of the surface is fatal to somatic cells from various animal tissues, although a puncture of the surface may be survived. Seifriz (1921) finds that vacuolar membranes from bread mould may persist and gel when the cytoplasm disintegrates. When a resting myxomycete plasmodium is torn, the last part to tear is always the outer border. An Amoeba, partially severed, may be drawn together again by the contraction of a thin remaining strand derived from the outer border. These results indicate the presence of physical differentiation in the outer layers of the protoplast. He describes (1927) removing the surrounding cytoplasm from the vacuolar membrane of the plasmolyzed onion cell. For the nucleate erythrocyte of *Cryptobranchus*, Seifriz (1926) describes an outer „wall" about 0.8 $\mu$ in thickness, which is moderately elastic.

Membranes surrounding the contractile vacuole in protozoa are of interest in view of Lloyd's report that vacuoles in the gamete of *Spirogyra* may function as contractile vacuoles (1928). Kite (1913 b) describes the wall of the contractile vacuole in Amoeba as of high consistency and adhesiveness, and the contractile vacuole walls of *Paramoecium* as of greater density than the endoplasm. Howland (1923) finds the wall of the contractile vacuole of *Amoeba verrucosa* and *Paramoecium caudatum* rigid enough to indent, rather than pierce, with a blunt needle. Taylor (1923) states that the contractile and subsidiary vacuoles in *Euplotes* may be moved about by needles, showing a wall of high consistency, and that shreddy remains of the walls of the subsidiary vacuoles may sometimes persist after the vacuoles have fused.

The evidence from unfixed material supports the idea of a nuclear membrane credited by cytologists. Kite (1913 a, b) describes definite nuclear membranes for marine ova, *Amoeba proteus*, and the epidermal cells of *Necturus*, although, he disagrees with the idea that most protoplasmic surfaces possess membranes. Chambers (1917) describes a definite nuclear membrane, which collapses and wrinkles when the nuclear fluid is withdrawn with a pipette, in the marine ova he has studied. Seifriz (1921) reports dissecting off the degenerate membrane from the dead nucleus of *Amoeba*. Scarth (1927) believes that the membrane of the nuclei in the plant cells on which he has worked is fluid in the living condition.

## THE INADEQUACY OF PRESENT EVIDENCE FOR THE EXISTENCE OF MEMBRANES AT PROTOPLASMIC SURFACES

Evidence from many different sources suggests a definite differentiation of the layers of protoplasm in the living cell which are in contact with cell wall, cell sap, and with the nucleus, or, in the last case, possibly differentiation of the surface layer of the nucleus itself. Certain points render this evidence insufficient for concluding that such differentiation regularly occurs in the living plant cell. It is still uncertain to what extent the organization of the protoplast in the walled, vacuolate plant cell parallels that in the animal cell. With the exception of Price's darkfield studies, evidence for plant material is not based on living protoplasts.

The following report is offered as a contribution to our knowledge of differentiation of surface layers in the living, normal plant protoplast.

## TERMINOLOGY

In discussing the literature, the terminology of the papers under consideration was largely followed, but considerable confusion exists. De Vries called the layer surrounding the vacuole the tonoplast and that at the outer surface of the protoplast, the ectoplast. Pfeffer referred to the two as plasma membranes. From the first, some difficulty has arisen from the occasional use of the word "membrane" as synonymous with cell wall. Now the common use of the word "membrane" alone to designate an *osmotic* membrane makes it a physiological rather than a purely structural term.

De Vries' "tonoplast" presents fewer difficulties. It is this term which the present writer has used to denote a distinct layer of cytoplasm about the vacuole.

"Ectoplast", however, is widely used in a wholly different sense. In a myxomycete plasmodium or in a protozoan, the ectoplast or ectoplasm is a thick layer of cytoplasm which is certainly not the outer "plasma membrane" in either a structural or a physiological sense. Mast (1924) has given us the term "plasmalemma" for the thin external layer which is the "membrane" if an osmotic function exists. This term seems free from the difficulties presented by both "ectoplast" and "plasma membrane". It has seemed permissible, therefore, to extend its use to the botanical world, and to employ it to denote a distinct, differentiated layer on the outer surface of the plant protoplast.

Since plasmalemma and tonoplast are themselves cytoplasmic structures, we cannot refer to the layer of protoplasm between them simply as cytoplasm. It obviously is not ectoplasm. Endoplasm, on the other hand, would include the tonoplast. No term in use at present adequately distinguishes this portion of the cytoplasm. A new one seems necessary to avoid confusion. *Mesoplasm* is proposed as indicative of its position in relation to the other layers.

Plasmalemma and tonoplast, then, will be used hereafter to denote differentiated layers at the interfaces between protoplasm and wall and protoplasm and sap, respectively, and mesoplasm for that portion of the cytoplasm which lies between these layers.

## METHODS AND MATERIAL

Chambers (1924) describes micromanipulation methods in general, and Scarth (1927) and Seifriz (1927) describe the application of these methods to plant cells.

The material used for the greater part of this work was the inner (morphologically, the upper) epidermis of bulb scales of Bermuda onions, results obtained with other cells being so designated. This, when stripped off, gives a layer only one cell in thickness, save where occasionally a patch of large, colorless mesophyll cells clings to it. This provides exceptionally favorable material for microdissection. Sections of the stripped epidermis may be mounted in a hanging drop of tap water in the micromanipulation moist chamber, and the cells entered by horizontal needles which pierce the end walls. This is the method SCARTH has used with other plant cells in his work. More conveniently, one may employ the method described by SEIFRIZ and HÖFLER. Strips of epidermis are plasmolyzed in 18 percent (0.56 M.) sucrose. This reduces the protoplast to about one half its original volume. The protoplasts are well rounded away from the end walls after about twenty minutes in the plasmolyzing solution. A strip is then cut with a sharp razor, transversely to the long axis of the leaf, on a glass slide. The blade passes between the end wall and the protoplast in many cells, leaving the protoplast untouched and uninjured. This section of epidermis is mounted in a hanging drop of plasmolyzing solution on a cover slip which forms the roof of the micromanipulation moist chamber, the open ends of the cells pointing towards the open end of the moist chamber. If desired, the material may be held in place by a blunt needle with a vertical tip. A dissection needle with a horizontal tip now has access to the protoplasts through the open ends of the cells, without touching the cell wall. There is no limitation on the movement of the needle, and one obstacle to succesful use of these methods on plant material is thus done away with.

This method was used for the greater part of this work, but unplasmolyzed cells were also studied, to eliminate any introduction of error as a result of plasmolysis. The method can be used for thicker tissues by plasmolyzing thin sections, then cutting the sections transversely after plasmolysis.

## DISTINCT BEHAVIOR OF MESOPLASM AND PLASMALEMMA IN PROTOPLASMIC STRANDS

Definite indication of the existence of an outer layer distinct from the rest of the cytoplasm is obtained when a strand is pulled out from the surface of the plasmolyzed protoplast by the microneedle. At first both mesoplasm and plasmalemma follow the needle and cannot be distinguished from one another. For a short time, material from the

protoplast will continue to flow into the strand as the needle is drawn back, then this flow will cease, and the material composing the strand elongates as the needle moves. The length to which the mesoplasm can be elongated is not very great. When this limit is reached the mesoplasm rounds into droplets, while the plasmalemma persists as a slender thread connecting the droplets and forming a layer over each droplet (Fig. 1). If the needle is drawn still further back, the thread stretches and the balls are carried farther apart. If the needle is moved towards the protoplast again, the strand contracts and the balls reapproach one another. They maintain their globular form until actually in contact. If the globules and the connecting thread were composed of the same material, we should expect to see the same shapes shown as the globules reapproach one another which were shown as they separated and rounded up, just as

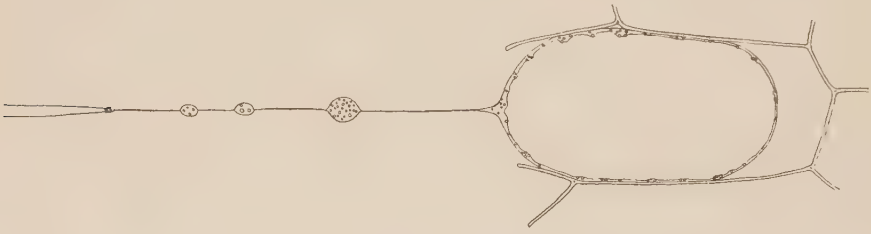

Fig. 1

an elongated soap bubble passes through the same forms when allowed to shorten as it did when elongated. But no sign of fusion of the droplets appears, until they are actually in contact with one another. Similarly, a globule reapproaching the protoplast itself must actually come into contact with the mass from which it arose before it loses its spherical shape and starts to flow back into the plasma mass, even though the two are connected at all stages by a thread of plasmalemma.

It has been known for some time (Chodat 1911) that when mesoplasm is included in strands persisting between protoplast and wall on plasmolysis, the cytoplasm rounds into balls on a thin thread which appears to arise from the outer layer of the protoplast. The behavior of the protoplasm on micromanipulation is evidently another manifestation of the same tendency. The conclusion that the outer layer and the inner cytoplasm are distinct seems inescapable. When a drop of homogeneous fluid is elongated until surface forces cause it to separate into droplets, these droplets are wholly free from one another; no connecting thread will remain. A highly viscous fluid such as tar or molasses

may be pulled out into a long, slender thread, but there will not be droplets on this thread. The layer forming the thread and the layer forming the balls must be distinct.

## PLASMALEMMA IN THE DEAD PROTOPLAST

The smooth surface of a dead, swollen protoplast can actually be torn away by the microneedle (Fig. 2) allowing the mesoplasm to flow out into the surrounding medium as a shreddy, granular mass which does not mix with the water. This brittle external layer is the structure which PFEFFER ruptured in acid-killed cells by deplasmolysis, allowing dyes to penetrate through the cracks so formed into the mesoplasm. It seems to retain some of the differential permeability of its living condition, but otherwise is very different from the fluid, extensile living layer from which it arose.

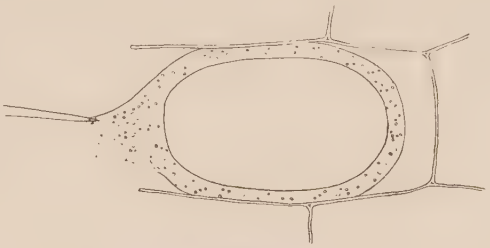

Fig. 2

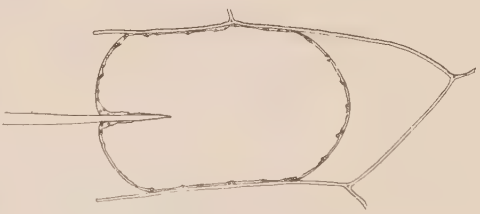

Fig. 3

Swelling of the protoplasm follows the death of unplasmolyzed as well as plasmolyzed cells. Since the protoplast of an unplasmolyzed cell is in contact with the wall, it is impossible to determine whether the dead plasmalemma can be torn away from the mesoplasm, but if a needle is pushed throug hthe wall until it pierces the outer layer but not the tonoplast, the swollen protoplast will collapse until the tonoplast lies almost flat against the wall. Apparently the dead plasmalemma held back the fluid which distended the mesoplasm, and its puncture allowed the fluid to escape.

It is no easy matter to puncture or tear the plasmalemma of the living protoplast. Even a very sharp needle carries a layer of protoplasm with it as it enters the cell; the needle invaginates, rather than pierces, the protoplast (Fig. 3); the plasmalemma lies next the needle, and the mesoplasm and tonoplast are also indented and carried in. When the needle is pushed through the protoplast until it traverses the vacuole and passes through the opposite side, the second protoplast wall stretches

and covers the advancing needle like a tent. It is evident from the movement of the granular mesoplasm that material for this cone and for the layer covering the needle is supplied by flow from other regions, but if the needle is pushed through far enough, particularly if it is moved rapidly, this flow will be inadequate and a strain will result which may find relief in one of several different ways. The plasmalemma may be ruptured, resulting in death of the cell. The protoplast may be completely punctured at the point at which the needle enters and at the opposite side where the needle tip stretches it into the form of a cone. In this case the severed edges of the layer about the needle come into contact with the edges of the puncture in the cone and fuse. The protoplast resumes it normal contours, but a tube of protoplasm now surrounds the needle which joins the protoplast at both ends (see Fig. 4). Perhaps the situation may be more clearly visualized by analogy with invertebrate embryology. The needle first invaginated the protoplast, resulting in a gastrula stage, the end of the "archenteron" was then brought into contact with the opposite wall of the gastrula, and an opening, the "stomadaeum", was formed. The vacuole corresponds in position to the coelom, and is at all stages completely shut off from the outside solution. This interpretation of the state of affairs can be corroborated when the needle is moved backwards and forwards. Material from the needle flows into the wall of the protoplast, and material at the other end of the needle flows from the wall of the protoplast onto the needle. In addition to these two possibilities, the strain produced by the needle may have a third result.

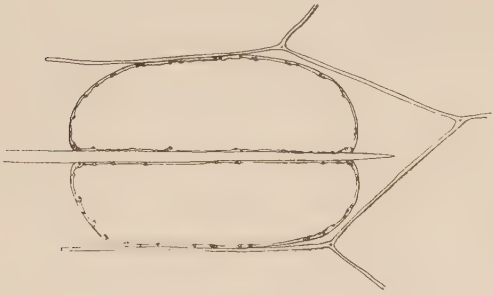

Fig. 4

## PUNCTURE OF THE VACUOLE AND CONTRACTION OF THE PROTOPLAST

Sometimes the layers of protoplasm about the needle remain intact while those on the opposite side of the protoplast, stretched by the needle, are ruptured. The opening enlarges rapidly, forming a gaping aperture. The cell sap is in direct contact with the external solution, and in the case of cells whose sap contains colored pigment, its outward diffusion

can be followed. The hole grows slowly after its initial rapid formation, and as it enlarges the protoplasm flows together until finally only a ball of protoplasm remains, containing a nucleus but no central vacuole (Fig. 5). What is now the outer layer of this mass consists in part of the layer originally in contact with the cell sap. The cytoplasm is normal, the streaming of the granules continues, and no swelling or appearance of Brownian movement results.

An enlarging puncture of this sort not infrequently arises at the point at which the needle first enters the protoplast, or more rarely,

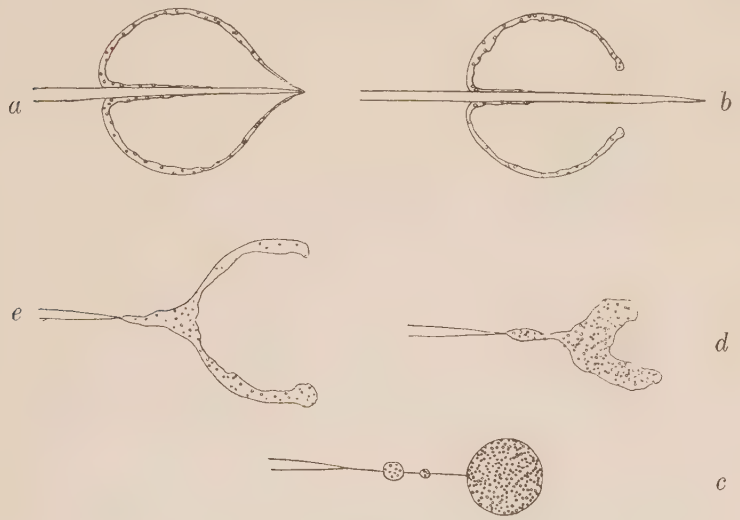


Fig. 5

at a point remote from the needle as a result of the tension produced. The last case is more common among cells which have stood for some time in plasmolyzing solution. This suggests that the internal structure of the protoplast undergoes gradual readjustment to its new form, and loses in extensibility. Enlarging punctures, not resulting in death of the cell, also occur at times in unplasmolyzed cells.

## ELASTICITY AS A CAUSE OF PROTOPLASMIC CONTRACTION

The fact that the hole formed enlarges so slowly after its first appearance suggests that its enlargement and the contraction of the protoplasm may be due to elastic forces rather than surface forces. It is hard to see why the rate of enlargement should change so abruptly if it were

the result of surface tension. Elastic substances, however, often show lag in recovery, returning rapidly partway to their original form, then completing the return very slowly.

There are other points in the behavior of the protoplast which are more readily explained on the assumption of elasticity than of surface tension alone. When a strand is pulled out from the surface of the protoplast and stretched farther and farther, it will finally break, and contract very rapidly, almost snapping back, and usually crumpling as it recoils (Scarth, 1927, has described similar behavior of internal cytoplasmic strands when broken.) These strands lag in the final stages of their contraction. The return of the final lagging portion to the plasma mass can be accelerated by pressing on the adjacent portion of the protoplast.

## ELASTICITY IN THE PLASMALEMMA

If a strand is pulled out from the protoplast but not elongated to the point at which the mesoplasm separates into balls, the heavy granular mass will at first flow back into the protoplast when the needle reapproaches it almost as fast as the needle itself moves, but at the last will flow more slowly, so that a portion persists for a time as a round protrusion on the surface of the protoplast. This will also flow back into the main mass in time, and can be made to flow back instantly by pressing with the needle on an adjoining portion of the protoplast. Now, such pressure indents the surface of the protoplast, making its curvature more sharp. When one liquid droplet flows into another as a result of surface tension forces, the speed of flow will increase as the difference between the curvature of the two droplets increases. Hence, the effect of such pressure should be to retard the flow, were it caused by surface tension. Again there is a strong suggestion that it is elastic forces, probably in the plasmalemma, that cause the flow. If the elasticity were resident in the mesoplasm, strands of varying thickness should flow with equal rapididity, whereas thicker strands flow more slowly than those in which the proportion of mesoplasm to plasmalemma is lower, and thin strands arising from the plasmalemma alone and carrying balls of mesoplasm move most rapidly of all.

## ELASTICITY IN THE CYTOPLASM

The behavior of the mesoplasm suggests that it, too, possesses elasticity in a low degree. It will be noted that when a strand is pulled out from the protoplast, some lines of granules appear to snap and flow

back towards the protoplast before others on either side of them do. This and similar phenomena when the needle is moved through the protoplast are explicable if we think of the mesoplasm as based on a continuous, labile, elastic framework. Some fibrils in such a framework might be under greater tension than other, and break and contract sooner. Such a structure has been suggested by Seifriz (1924, 1929) and supported by Scarth (1927).

## TEARING THE LIVING PLASMALEMMA

The layer of protoplasm in the plasmolyzed or unplasmolyzed cell is so thin that it is difficult to demonstrate that tearing the outer surface has wholly different effects from piercing the entire thickness of the protoplast. But if one of the contracted protoplasmic balls resulting from a widening puncture of the protoplast is used, it can be seen that the needle may pierce the entire thickness of such a ball without injury, while if the outer surface is town with a rapid motion, the mesoplasm swells and ceases streaming, the swelling commencing at the ragged edge of the tear (Fig. 6).

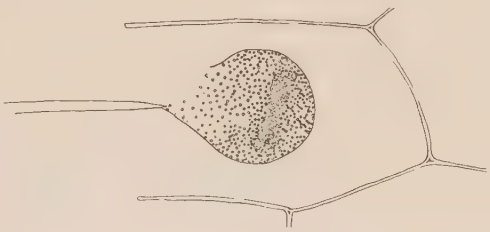

Fig. 6

## WHAT IS THE SIGNIFICANCE OF STRANDS PERSISTING BETWEEN PROTOPLAST AND WALL AFTER PLASMOLYSIS?

Küster (1910) has noted that portions of a protoplast separated by plasmolysis frequently do not fuse on deplasmolysis. This fact is occasionally cited as evidence for the existence of a distinct external layer on the protoplast. This is questionable, for two soap bubbles may likewise fail to fuse when brought into contact, and the two portions of the protoplast, filled with and surrounded by liquid, are more analagous to soap bubbles than to liquid droplets. The behavior of isolated portions of protoplasts brought into contact with one another could be further followed here. If the solution in which plasmolyzed, sectioned material is mounted is diluted, the protoplasts will move toward the open end of the outer cells as they swell, until they partially protrude or are even set free in the surrounding medium. A partially protruding protoplast can

be pinched in two with the needle, much as a soap bubble can be divided, but in this case a thin strand of plasmalemma connects the two portions (Fig. 7). One portion is anucleate, yet streaming in it will continue in exactly the same manner as in protoplasts containing the nucleus, even after the connecting thread is broken. A ball of protoplasm thus separated will not fuse when brought again into contact with the other portion of the same protoplast or with another protoplast, which confirms KÜSTER's observation. However, when the two balls of protoplasm are separated again, a newly formed plasmalemma thread will connect them. These threads suggest plasmodesmae. It is already known that plasmodesmae must arise secondarily, since they occur between cells of different genetic origin in graft hybrids (HUME 1913), but whether or not there is true

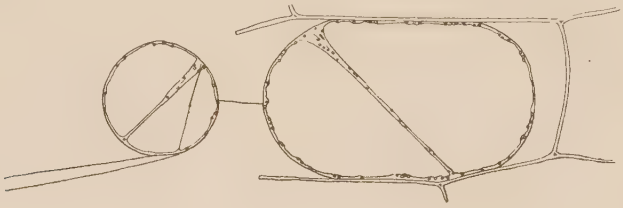

Fig. 7

cytoplasmic continuity in plasmodesmae is still an open question. The threads seen here originate from a secondary contact of protoplasm, and apparently involve no true fusion of mesoplasm.

Strands running from a plasmolyzed protoplast to the cell wall are sometimes thought to arise from plasmodesmae. Others suggest that their presence indicates the continuity of substance between wall and membrane which CRANNER (1919) has supported. The greater number of the strands disappear after prolonged plasmolysis. The few that remain can be broken by careful manipulation. But whenever a free protoplast is brought into contact with the wall, a new strand will form at the new point of contact. In order to remove the protoplast this new strand must be broken, and in breaking the first a new contact is apt to be made and another strand formed, and so on. These newly formed strands fasten the protoplast to the wall as securely as those persisting from plasmolysis. In one case a plasmolyzed protoplast, lying in a cell which had both end walls removed, was rolled over and over by two needles from one end of the cell to the other, all previously existing strands thus being broken. The plasmolyzing solution was then made more concentrated,

and on further contraction of the protoplast it was evident that numerous new strands now connected it with this new region of the wall. When we also consider the fact that the strands between needle and protoplast are fully as persistent as those between protoplast and wall, it seems that the strands are more likely the result of the glutinosity of the plasmalemma than of continuity of substance between wall and protoplast, or of the presence of plasmodesmae.

## THE TONOPLAST

The tonoplast is the most striking of the differentiated protoplasmic layers in that it can be completely isolated and still retain many of the properties shown in the living cell.

### DISTINCTNESS OF THE TONOPLAST IN THE LIVING CELL

The best indication of a distinction between tonoplast and mesoplasm in the normal cell is obtained when a needle draws out a strand from the wall of the vacuole. Frequently the granular material in such a strand will round up into balls on a hyaline, tonoplast thread, much as the mesoplasm rounds into balls on an external plasmalemma thread when a strand is pulled out from the outer surface. The fact that this does not invariably occur is probably due to the fact that an internal strand cannot be elongated to any great extent, whereas a thread pulled out from the external surface can be elongated to several times the length of the protoplast, if necessary, in order to reach the limit of extensibility of the mesoplasm.

On the death of the cell, the material composing the transvacuolar strands, as well as the remainder of the cytoplasm, swells. The mesoplasm in the strands flows together to form globules on a thin, hyaline thread. Threads which originally contained no granular material do not swell visibly, and show no rounding into droplets. Apparently the material composing the tonoplast swells little, if any, on death.

### ISOLATION OF THE TONOPLAST

The tonoplast will often remain intact when a sudden, tearing motion of the needle ruptures the plasmalemma. The mesoplasm swells and contracts, slipping back over the surface of the tonoplast (Fig. 8). Plasmalemma and mesoplasm can be completely removed by the needle, leaving the tonoplast free, as a transparent bag filled with cell sap. The

tonoplast is clear, colorless, hyaline, apparently quite fluid and only slightly sticky. The isolated vacuole swells in a diluted medium and shrinks in a concentrated one. If it contains colored sap (as in cells of the beet, occasional cells of *Elodea* and onion) it is evident that the pigment is retained. If placed in a colored solution, the dye does not penetrate. Since this layer retains its differential permeability, it seems not unlikely that its physical condition may also closely approach that of the same layer in the normal cell. When the tonoplast is elongated by the needle, it breaks or separates, like an elongated soap bubble, into small globules containing cell sap. Only short strands can be pulled from its surface. Torn, it flows together into a transparent mass. In the isolated tonoplast we have a striking instance of a fluid membrane possessing differential permeability approaching that of the living protoplast.

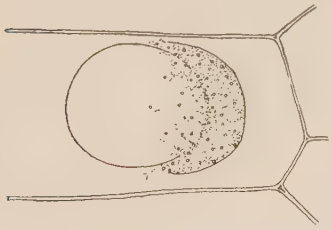

Fig. 8

Separation of the tonoplast on death also occurs in cells mounted in tap water, so its isolation cannot be attributed to the plasmolyzing agent. In non-plasmolyzed cells cut by the razor in sectioning, the tonoplast forms a layer distinct from the swollen mesoplasm, and can easily be separated with the needle. The separation of tonoplast from mesoplasm can be followed as an unplasmolyzed protoplast swells as a result of injury with the needle. Starting at the point of injury, the tonoplast appears to blister off in a manner recalling the separation of the fertilization membrane from an echinoderm egg. At times, the tonoplast is not, at first, distinct from the swollen mesoplasm, but becomes distinguishable later as the mesoplasm shrinks away and collapses against the plasmalemma and wall. The tonoplast isolated from unplasmolyzed cells behaves on manipulation just as does the same layer from plasmolyzed cells.

The number of different ways in which separation of the tonoplast can be brought about is striking. If plasmolysis or deplasmolysis is very rapid the tonoplast may separate from the rest of the cytoplasm. If the protoplast is cut by the razor, the tonoplast may separate while the rest of the cytoplasm swells and flows together. If an injection or the insertion of a needle produces too great a strain in the cytoplasm, the outer layers may be ruptured or swell, and the tonoplast persist. If the nucleus is injured, the tonoplast will remain distinct while the rest of the protoplasm undergoes marked changes. Certainly a layer which appears in both plasmolyzed and unplasmolyzed cells, whether death occurs slowly

or suddenly, whether death is caused by mechanical injury or by toxic products of nuclear injury, which is invariably distinct from the remainder of the protoplasm and which always possesses the same properties, must represent a layer distinct from the remainder of the protoplast in the living condition. Taking into consideration the difference in behavior of the granular and hyaline material when a strand is pulled out into the vacuole from the vacuole wall, it seems certain that the living protoplast possesses a differentiated layer about the vacuole.

## THE NUCLEUS

The nucleus in the onion cell is irregular, usually flattened and roughly disk shaped. This flattened form persists even when the nucleus is suspended in the middle of the vacuole rather than lying in the peripheral layer of cytoplasm. Many nuclei show deep, transparent grooves which occasionally pass completely through the nucleus (Fig. 9). The nuclear substance presents a finely mottled, apparently alveolar structure. Two nucleoli occur.

Fig. 9

## ELASTICITY OF THE NUCLEUS

The suggestion of elasticity and rigidity which is given by the irregular shape of the nucleus is confirmed by its behavior when handled with microneedles. The nucleus may be pushed slowly from end to end of the cell without changing shape, and if a disk—shaped nucleus which is in such a position that it appears circular is caught on one edge by the needle tip, it will be tipped up and appear as a narrow ellipse. When the needle is withdrawn it falls into its original position and again appears circular.

If greater force is applied, the nucleus may be distorted, but resumes its shape with striking regularity. When pushed rapidly through the cytoplasm, the pressure of the cytoplasm against its free edges will bend the nucleus until, in profile, it is sausage shaped. The nucleus springs back to its original shape when the needle is brought to rest. This experiment may be repeated many times, and the nucleus invariably straightens out.

The return of the nucleus to its original form cannot be the result of surface tension forces, since the form which it resumes is flattened and highly irregular, in no way approaching the spherical form towards which these forces would work.

Fig. 10 shows the return of a living nucleus to its original form when distorted by the needle. The nucleus was so irregular in contour that the needle tip could be hooked under one of the protrusions and pulled laterally, producing marked distortion, yet after each of several successive distortions the form resumed was very close to the original.

One protoplast afforded a demonstration of elasticity in the nucleus without a needle touching the latter. Occasionally, when a needle pierces a protoplast and passes on through the end wall of a cell, the protoplast will flow through the hole left after the needle is withdrawn. This is probably a result of suction, the wall of the second cell being somewhat stretched as the needle is withdrawn. Flow can take place through a hole so small as to be perceptible with great difficulty. The protoplasm which passes through the hole remains alive, for active streaming takes

Fig. 10

place. In the case under consideration, the protoplast continued to flow through the hole into the adjoining cell until the nucleus was carried against the hole, passed part way through, and stopped. The hanging drop was then diluted, and the expansion of the protoplast in the second cell now forced out the intruding protoplast. The nucleus sprang back, and resumed its original form. When the concentration of the drop was increased, the first phenomenon was repeated, and once more the flow stopped when the nucleus plugged the hole. Again dilution of the drop forced the protoplast back, and again the nucleus resumed its original form. Its rigidity was evidently too great to allow it to pass through the opening through which the protoplasm flowed so easily.

## CHANGES IN THE DYING NUCLEUS: THE NUCLEAR MEMBRANE

The nucleus undergoes striking and instant changes when the cytoplasm is torn or when the nucleus itself is subjected to sudden pressure. The first sign of death is the disappearance of the mottled structure; this is followed by swelling. As the nucleus thus changes, a death wave passes over the cell, originating at the nucleus and becoming evident

in cessation of streaming and swelling of the cytoplasm. The nucleus continues to swell until almost spherical. The contents of the two nucleoli form granular masses. In a cell which has been plasmolyzed the nucleus may remain in the rounded, hyaline form for some time. In an unplasmolyzed cell, the mottled appearance reappears almost immediately after the nucleus becomes spherical. The marking may be even more distinct than in the living nucleus, but is coarser. The smooth, spherical form and the granular nucleoli are clearly unlike those of the living nucleus. In the plasmolyzed cell these changes may be deferred for several minutes after swelling. After the mottled pattern reappears, shrinking sets in, slowly in the plasmolyzed cell, but very rapidly in the unplasmolyzed cell. In the latter the collapse is very irregular, the nucleus shrinks in some spots until concave, and from these a membrane lifts off (Fig. 11). It is impossible to say whether this membrane originates from the cytoplasm or from the nucleus. It is something more definite than the result of shrinkage, separating nucleus and cytoplasm, for when the nucleus is pulled out of the cytoplasm by the microneedle, the blistered membrane goes with it.

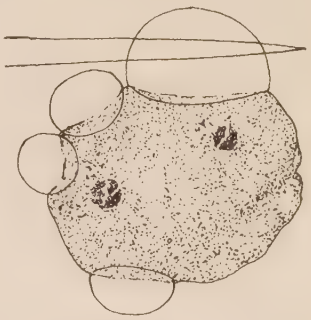

Fig. 11

If the nucleus is roughly handled with the microneedle while swelling, the reappearance of mottled structure and the commencement of shrinkage are brought on at once—before swelling is complete in unplasmolyzed cells, or without the lapse of time, after the first stages of swelling, which intervenes in plasmolyzed cells.

## THE NUCLEUS AS AN OSMOTIC SYSTEM

The following explanation is offered for the behavior of the dying nucleus. The nucleus is surrounded by a differentially permeable membrane. Swelling is a result of the action of acid (from injury) on the nuclear proteins. At a certain point, swelling stretches the membrane until its differential permeability is lost. (Similar suggestions have been made for the loss of haemoglobin at a certain stage in the swelling of erythrocytes.) At this point electrolytes from the cytoplasm and surrounding medium penetrate the nucleus, and the proteins are "salted out", bringing about the reappearance of the mottled structure. Shrinkage (syneresis) follows. The reason that a longer interval elapses before

the mottled structure reappears in plasmolyzed cells is that the higher external osmotic concentration retards the last stages of swelling. Rough manipulation with the needle ruptures the membrane and allows the electrolytes to enter immediately. In the case of the unplasmolyzed cell, puncturing the membrane allows the electrolytes to enter before swelling has gone far enough to destroy the semi-permeability of the membrane. A combination of colloidal and osmotic theory seems necessary to account for the behavior of the nucleus.

## ELASTICITY OF THE DEAD NUCLEUS

The dead nucleus, removed from the cell and stretched between microneedles, shows marked elasticity. It may be stretched to five times its original length and contract to a little over twice its original length. It if is stretched until its elastic limit is reached (10—15 times its length) it tears and snaps back as it contracts, but is now merely a fibrous mass clinging to the needle tips.

## CONFIRMING RESULTS FROM OTHER CELLS

Leaf cells of *Elodea*, cortical parenchyma cells of beet and carrot roots, mesophyll cells from the cabbage leaf, cortical parenchyma cells from stems of young seedlings of *Lupinus albus*, and root hairs of *Trianea* were used as material to supplement the work on onion cells.

Separation of the tonoplast as a result of mechanical injury was seen in the first four named, and in the last, but did not occur in the cells of *Lupinus*.

The formation of balls of mesoplasm on a thread of plasmalemma was seen very clearly in *Elodea*, both in strands persisting between protoplast and wall after plasmolysis and in strands pulled from the outer surface of the protoplast with the needle. In *Trianaea* root hairs, such balls could be seen on strands persisting after plasmolysis and also when strands were pulled out with the microneedle from the surface of living, extruded cytoplasm. Balls appeared regularly on strands pulled out from the protoplast of the carrot cells, but the smallness of the protoplast and the thinness of the layer of cytoplasm make it impossible to say with certainty, as can be said in the other cases, that a part of the vacuole was not carried out into the strand pulled out from the protoplast, and that its separation into smaller vacuoles was not the cause of the formation of balls.

The failure of such balls to form in cabbage, beet, and lupine cells is connected with the fact that the strands pulled from the surface of these protoplasts are short, ordinarily breaking and starting to contract before they have exceeded the length of the protoplast.

In all the cells, contraction of the strand after breaking resembles contraction of an elastic body rather than flow of a true fluid, being much more rapid in the first than in the last stages. Contraction is always accelerated by pressure on the protoplast.

Cells of several types showed that puncture of the entire thickness of the protoplast, followed by contraction of the protoplasm into a ball, was not fatal. This could be seen most clearly in *Elodea*.

In no case did liberated cytoplasm mix with water. Its behavior can be followed best in the hairs of *Trianaea*. Here cytoplasm which is forced out violently swells at first, and some particles enter into Brownian movement. The mass may look as if it were composed of discrete particles, but when a needle is thrust into it and retracted, the entire mass is pulled out after the needle, finally breaking and contracting. Evidently a continuous structure persists, even though not visible. The swollen protoplasm shrinks and darkens soon after liberation. It then behaves like a fairly rigid body when pushed about with the needle.

## CONCLUSIONS

*The internal and external surface layers of the protoplasm, of onion cells indistinguishable microscopically or ultramicroscopically, are nevertheless distinct from the rest of the cytoplasm.* The corroborative evidence from other cells suggests that a distinct plasmalemma and tonoplast, ordinarily too thin to be detected optically, are regular features of the organization of the plant protoplast.

*The plasmalemma is protective in function.* This is shown by the fact that it is fatal to tear this surface layer, although the cell is not ordinarily injured by prolonged probing. The fact that the protoplast may be punctured as a whole, opening the vacuole to the outside soloutin, without fatal effects, suggests that the tonoplast affords protection to the mesoplasm similar to that given by the plasmalemma.

*The plasmalemma is highly elastic. Elasticity is less marked, but detectable, in the mesoplasm. The nucleus is the most highly elastic of the components of the onion protoplast.* The protoplasm thus behaves as though based upon the continous structural framework of a gel, rather than

upon a continuous aqueous phase like that of an emulsion or suspension. This is in agreement with the familiar fact that rapid change in configuration of the protoplast (plasmolysis, deplasmolysis, pressure) is ordinarily fatal even though the same result, produced by gradual steps, can be brought about without injury.

Plasmalemma and tonoplast are elastic fluids. Noting the extensibility of the plasmalemma, the ease with which it accommodates itself to changes in configuration, the fact that the greater part of a protoplast may flow through a minute opening without injury, a new explanation suggests itself for the phenomena first noted by Nägeli (1855) and repeatedly confirmed and cited since. It has generally been considered, in agreement with his account, that the fact that exuded or expressed protoplasm from root hairs, pollen tubes, *Vaucheria*, etc., behaves as if surrounded by a membrane wholly similar to that of the intact protoplast is an indication that the protoplasmic membrane is "autonomous", or that the formation of surface membranes by protoplasm is a result of surface forces at the protoplasm-water interface. Is it not likely that in these cases we are *not* dealing with the reformation of a membrane by naked mesoplasm, but that plasmalemma as well as mesoplasm is forced out, and that the mesoplasm is covered, at all stages, by the fluid plasmalemma? The droplets which fail to "re-form" a membrane are then those which consist of mesoplasm with no covering plasmalemma.

## SUMMARY

1. Existing evidence for the formation of morphological membranes at protoplasmic surfaces in the living plant protoplast is reviewed and judged inadequate.
2. Cases from the literature and from the author's work are cited to show that micromanipulation may be employed in the investigation of this problem without perceptible disturbance of the normal condition of the protoplast.
3. Investigation of plasmolyzed and unplasmolyzed protoplasts with the microneedle indicates:
   a) That there is an external layer, or plasmalemma, surrounding the protoplast, which, while fluid, is more elastic and more extensile than the remainder of the cytoplasm.
   b) That a similar, but less elastic layer, the tonoplast, surrounds the vacuole.

c) That the mesoplasm is much less extensile than the tonoplast and plasmalemma between which it lies.

d) That the nucleus is surrounded by at least one membrane of either cytoplasmic or nuclear origin. The nuclear material, while highly elastic and showing the properties of a hydrophilic colloid, nevertheless is part of the osmotic system which the entire nucleus constitutes.

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# MEMBRANES IN THE PLANT CELL

## II. LOCALIZATION OF DIFFERENTIAL PERMEABILITY IN THE PLANT PROTOPLAST

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In part one of this paper (PLOWE, 1931) evidence was given indicating that the living plant protoplast possesses differentiated layers, the plasmalemma and the tonoplast, at the cytoplasm-wall and cytoplasm-sap interfaces. The following study was made with the purpose of determining whether or not the differential permeability possessed by the plant protoplast during life is localized in these physically distinct layers.

It does not, of course, necessarily follow that the entire thickness of these layers functions as an osmotic membrane, even if it can be shown that the differential permeability of the protoplast is a property of tonoplast and plasmalemma and not of the mesoplasm. This distinction will, however, be waived for the present, and discussed after the major problem has been taken up.

From the results of countless experiments on record, a few conclusions may be drawn which are apparently applicable to any living cell. Water can move readily both into the cell and out. The movement of water is influenced by the composition of the external milieu. Solutes as a rule move less freely than water, and between different solutes, such as urea and sucrose, there may be wide differences in the ease with movement takes place. Certain substances, such as basic dyes, may move into the cell readily but move out of it with infinite slowness, so that an appearance of "one-way permeability" results.

In the greater part of the mass of work on cell permeability, the permeability of the cell to a certain substance is considered synonymous with penetrability by that substance. It has been recognized in a smaller number of cases that ability of substances to pass out of the cell must also be considered.

Two basic theories have been advanced to explain the relation of the cell to water and solutes. The first, and classical, theory is that the cell is surrounded by a differentially permeable membrane, and thus constitutes an osmotic system. The second theory is that the intake and output of water and solutes by cells are the results of hydration and adsorption in the colloidal protoplasm. Those who advocate the second explanation do not claim that the osmotic concept is invalid for vacuolate plant cells, where the colloidal protoplasm itself would function as a differentially permeable membrane around the vacuole; but they maintain that the relations of this protoplasmic layer with external medium and vacuolar sap are colloidal, not osmotic.

Our present concern is not the specific permeability of the protoplast for different solutes, or the mechanism by which membranes function, or the details of hydration and adsorption. The question under consideration is: Is the permeability of the surface layers of the protoplast the limiting factor in the permeability of the cell?

Three pictures of the organization of the vegetative plant cell can be suggested:

1. A layer of differentially permeable protoplasm surrounding a vacuole.

2. A differentially permeable membrane at the outer surface of the (comparatively) freely permeable mesoplasm, with the vacuolar sap in direct contact with the mesoplasm.

3. Differentially permeable membranes at both outer and vacuolar surfaces of the (comparatively) freely permeable mesoplasm.

Kite's microinjection studies on the permeability of plant cells (1913a, 1915) led him to the conclusion that there is no essential difference between the permeability of internal and external cytoplasmic layers. Pfeffer (1897) as already mentioned, demonstrated localization of differential permeability in the coagulated plasmalemma of the acid-killed *Hydrocharis* root hair. With these exceptions we have no *direct* evidence either for or against differentially permeable membranes in the plant cell. Considerable indirect evidence is to be had on either side of the question, but it must be remembered that inevitable osmotic effects in the vacuolate plant cell add to the difficulty of correctly interpreting indirect evidence.

## THE RELATION OF THE CELL TO WATER AND SOLUTES ON A COLLOIDAL BASIS

Lloyd (1915, 1917) finds that acids and alkalies, in concentrations too low to be osmotically effective, first increase, and in higher concentrations decrease the rate of growth of pollen tubes in sugar solution. He believes this to be an indication that the swelling of growth is a hydration phenomenon rather than an osmotic one. The fact

that it is possible to explain these results as LLOYD does without hypothecating a differentially permeable membrane should not obscure the fact that it is equally possible to explain them on a similar basis in the presence of a membrane. Changes in the swelling capacity and osmotic pressure of gelatin run closely parallel, according to LOEB (1922). It might easily be conceived that the acid used by LLOYD altered the osmotic pressure of the cell proteins within a membrane, and that the volume change was osmotic. Use of acids of different penetrating power at the same pH might throw some light on the subject, since, if the osmotic explanation is the correct one, it is the effect on internal, rather than external, pH which is of importance.

Purely colloidal permeability theories have been advocated by workers on animal tissues. MARTIN FISCHER (1907, 1908) and D. J. LLOYD (1916) suggest explanations of volume changes in animal tissues in terms of protein hydration, the first pointing out the parallel effect shown by different acids and neutral salts on the swelling of fibrin and of animal tissues, and the second, the failure of the volume changes of muscle in acid and basic solutions to follow osmotic laws. MOORE and ROAF (1908) and MOORE, ROAF and WEBSTER (1911) propose to discard the employment of membranes in explanation of the distribution of solutes between plasma and blood corpuscles, substituting the idea of the formation of loose chemical compounds and adsorption products. But SIEBECK (1912) and BEUTNER (1913) find that volume changes in animal tissue due to the effect of acid are distinct from demonstrable osmotic effects present at the same time.

BAKHUYZEN (1928) advances a combination of adsorption and hydration theories to explain water intake, penetration of solutes, growth, excretion, and accumulation of substances inside the cell, pointing out, on data from the literature, that the HOFMEISTER series holds for the penetration of cells by ions.

## WATER AND SOLUTE RELATIONS AS OSMOTIC PHENOMENA

Evidence supporting the membrane theory for plant cells is wholly indirect. Although the behavior of plant cells in plasmolyzing solutions was the starting point of osmotic theory, our lack of knowledge of non-vacuolate cells (and DANGEARD, 1927, says "Il n'y a pas de cellule sans vacuole") prohibits us from rigidly terming the plant cell an osmotic system unless we limit the term to the relations between vacuolar sap and external milieu.

The high electrical resistance of plant tissues (OSTERHOUT, 1922) has been attributed to impermeability of the surface layers to electrolytes. Such an interpretation for resistance in animal tissues is corroborated by the demonstration of HÖBER (1910) and MCCLENDON (1926) that free electrolytes are actually present within the cell. HÖBER shows that erythrocytes placed in the dielectric between the two plates of a condenser affect the capacity in the same manner as a conducting substance in the same position. MCCLENDON finds that the resistance to passage of current decreases as the frequency of an alternating current employed increases and the path which ions travel in carrying the current becomes shorter. Neither method would serve to distinguish decisively between electrolytes in the vacuole of plant cells, unable to pass through the protoplast, and electrolytes in the protoplast unable to pass through a membrane. REMINGTON (1928), using MCCLENDON's method on beet root tissue, assumes, in order to interpret his results, that each cell possesses a single plasma membrane.

## THE ANIMAL CELL AS AN OSMOTIC SYSTEM ON THE BASIS OF MICROINJECTION EXPERIMENTS

There is direct evidence in support of the existence of differentially permeable membranes in animal cells. KITE (1913) shows that injected dyes may diffuse readily through the cytoplasma of *Amoeba*. CHAMBERS (1924) shows that many dyes which do not penetrate Amoebae and sea urchin eggs on immersion can diffuse readily through the cytoplasm on injection. In the case of the ova he first reported that cytolysis accompanied the diffusion of the dye, and some question was raised as to whether the permeability of the cytoplasm was not an effect of injury. Subsequent improvements in technique have done away with the difficulty. Today, microinjection is a standard method for the introduction of pH and rH indicators to which the cell is normally impermeable. (NEEDHAM 1926 a, b; CHAMBERS and POLLACK 1927; CHAMBERS, POLLACK and HILLER 1927; CHAMBERS 1928; RAPKINE and WURMSER 1926 a, b.) The viability of injected material is attested by normal movement of amoebae and lack of cytolysis in ova.

Demonstration by microinjection of localization of differential permeability at surfaces has not been confined to dyes. CHAMBERS (1922) finds that, while immersion in ammonium chloride or in sodium bicarbonate changes the color of starfish eggs vitally stained with neutral red to the basic and to the acid color respectively, injection of these solutions will cause change of color in the other direction. Using the explanation offered by JACOBS (1920, 1922) for the effect of immersion in solutions of $CO_2$ in sodium bicarbonate and of ammonium chloride on intracellular reaction, CHAMBERSs results are to be interpreted as showing that the hydrogen and hydroxyl ions which fail to pass through the outer surface of the cells from the external solution diffuse freely through the cytoplasm when the solution is injected.

There seems to be little question that the internal cytoplasm of the animal cells investigated is normally more permeable than the surface layers. Are we justified in concluding that since this is true for echinoderm eggs and amoebae and a few other animal cells, it is also true for plant cells? The gap is rather too wide to bridge without further evidence, and, even assuming that the two cases are analagous, leaves us still uninformed as to the nature of the relation between cytoplasm and vacuole.

## THE VACUOLAR MEMBRANE

Very little evidence is forthcoming on the permeability of the vacuolar membrane. In general, if there is an external membrane and the cytoplasm is freely permeable, it is necessary to assume that the vacuolar membrane is differentially permeable, in order to account for the failure of colored sap to color the cytoplasm, the close analogies between the properties of DE VRIES' tonoplasts and entire protoplasts (1885), and the anomalous contraction of vacuoles noted by OSTERHOUT (1913 a, b), KÜSTER (1926), and GICKLHORN and WEBER (1927). This is especially true of OSTERHOUT's observation that phycoerythrin set free from the plastids in the swollen cytoplasm of *Griffithsia* does not penetrate the vacuole. But, unless it is true that an external membrane exists, no one of these cases really tells us anything about the permeability of the vacuolar membrane, for in that case the first becomes but the logical result of the differentially permeable nature of the cytoplasm, and the last two represent retention of differential permeability by one interfacial layer longer than by the remainder of the injured cytoplasm.

Kite's work (1913a, 1915) leads him to believe that no differentially permeable membrane surrounds the vacuole.

Howland (1928) finds that the acid dyes of the Clark-Lubs pH indicator series do not diffuse into the cytoplasm of *Actinosphaerium* when injected into the food vacuole, but we cannot safely assume that a food vacuole in a rhizopod and a sap vacuole in a plant cell are analagous.

## Kite's EVIDENCE AGAINST PRESENCE OF DIFFERENTIALLY PERMEABLE MEMBRANES

Since Kite's experiments on plant cells give us our only direct evidence on the existence or non-existence of differentially permeable membranes in the plant cell, and since no careful consideration has hitherto been given to the direction as well as the extent to which injury may have influenced his results, his papers require further discussion before proceeding with the investigation of the problem.

Kite (1913a, 1915) reports investigations using a considerable number of plant cells. He concludes that the cellulose wall, and not the protoplasm, of plant cells prevents the entrance of dyes; from these results and his work on animal cells he concludes that "partial permeability to water, dyes, and crystalloids, is a property of *all* portions of protoplasmic gels". His conclusion, however, must be considered open to serious question for the following reasons. His own accounts indicate that in many cases the cells were injured by the injection, and in other cases there is no mention of any criterion used to decide whether or not the cells were injured. His intracellular injections in plant cells were always vacuolar injections, not cytoplasmic injections. No dyes were used to which the protoplast is impermeable. Consequently, although to Kite belongs the credit for pointing the way to direct experimental investigation of the question of differentially permeable membranes in the living protoplast, in order to find a satisfactory answer to the question it is necessary to elaborate upon his methods and above all to carefully distinguish between permeability as a result of injury and normal permeability.

## METHODS

Dyes which do not penetrate the cell immersed in them were injected into the vacuole, to determine whether the apparent impermeability on immersion was in reality the result of an unequal equilibrium distribution between external solution and vacuole, and into the cytoplasm, to ascertain penetrating power when passage through surface layers was not involved.

A Chamber's microinjection apparatus was used in connection with a Péterfi micromanipulator. Pipettes having the same form as the needles already described (Seifriz 1927) were used, with horizontal instead of vertical tips. The sealed ends were broken open against the end of the coverslip rather than the lower surface. When the horizontal pipette is used, there is no question as to the exact position of the tip

of the pipette at the time of injection, as there often is if one attempts to decide whether a vertical tip lies in the vacuole or in the cytoplasm. In work on animal cells there is not this difficulty to be met, and the simpler type is satisfactory. The material to be injected was held in place with a blunt, vertical needle.

Two questions arise with regard to the soundness of this method of investigating permeability. First, is it possible to distinguish accurately between a cytoplasmic and vacuolar injection? Second, can the behavior of a substance injected into the cytoplasm give information about the permeability of protoplasm in the normal condition? A convincing affirmative answer to the first question is obtained when the indicator dye brom cresol purple is used. Its color changes from the intermediate (half neutralized) color in the pipette to the acid yellow when injected into the vacuole, and to the basic blue when injected into the cytoplasm. In order to leave no doubt as to the answer of the second question, we may consider as normal only those cases in which streaming of cytoplasm is uninterrupted (in *Allium* and *Trianea*) or in which the easily disturbed normal configuration of the plastids remains unchanged and no Brownian movement appears (in *Griffithsia*). Further evidence of the normal condition of injected cytoplasm will be given later.

The cells used for this study were root hairs of *Trianea bogotensis*, epidermal cells of *Allium cepa*, and the coenocytic cells of *Griffithsia bornetiana*. The results for each will be taken up separately.

## ROOT HAIRS OF TRIANEA

The large root hairs of *Trianea bogotensis* offer good material for microinjection studies. The cytoplasm forms a fairly thick layer, and frequently masses temporarily at the tips of the hairs. Streaming continues within these masses. The wall is easily pierced. The absence of plastids, the transparency of the cytoplasm, the thinness of the walls, make internal details clearly visible. Injections into cytoplasm or vacuole can be made with no ambiguity as to location. The active streaming enables the observer to constantly check the condition of the protoplasm under observation.

## FAILURE OF DYES TO PENETRATE ON IMMERSION

Aniline blue, acid fuchsin, and the indicator brom cresol purple were used. The first two never penetrate the living cells. Cells immersed in one percent aniline blue for twenty four hours showed no color in cyto-

plasm or vacuole. Acid fuchsin is rather toxic, hence the longest immersion used was two hours forty five minutes in a 0.5 percent solution. In both cases the cells were plasmolyzed after removal from the dye, to avoid mistaking coloration of the wall for penetration of the protoplast by the dye, and to intensify any color which might be present in the vacuole if dye had penetrated. Shortage of the *Trianea*, aupply prevented immersion tests with brom cresol purple, but in no case was dye in the surrounding solution taken up by living cells.

## FAILURE OF DYES TO PENETRATE THE PROTOPLAST FROM THE VACUOLAR SAP

Aniline blue or acid fuchsin injected into the vacuole diffuses freely until the sap throughout the length of the vacuole is colored. The spreading of the dye is accelerated by the motion given to the sap by the streaming cytoplasm. It is interesting to note that the streaming of the cytoplasm is much more rapid than diffusion of the dye. Curtis (1928) maintains that streaming in phloem cells accelerates transfer of food substances, and the picture here seems to support the idea that protoplasmic streaming is a more rapid means of transportation than free diffusion.

When the pipette is withdrawn, a small amount of protoplasm flows out after it (very little if the cells are in isotonic, (0.068 M) $KNO_3$, solution) and the hole in the protoplast is this closed. If plasmalemma as well as mesoplasm is included, a globule is formed which is pinched off. If not thus protected, the mesoplasm coagulates.

Cells with dye in the vacuolar sap were preserved in hanging drops and observed from time to time to note whether the dye passed out of the cell or diffused into the cytoplasm. In no case did color appear in the cytoplasm. If any dye penetrated, the resulting concentration, compared with that in the vacuole, must have been very low. Aniline blue injected into the sap was retained in every case until after streaming had ceased and death changes in the cytoplasm set in. In one case, streaming continued and the color was retained for twenty hours after injection. The same statements describe all but three cases in which cells were injected with acid fuchsin. These (after three hours twenty-five minutes) had lost color from the vacuole, though streaming continued in most of the cytoplasm. The cytoplasm at the tips of the cells was abnormal in appearance, suggesting that local changes might have been responsible for the loss of color.

We may conclude that the protoplast of the *Trinaea* root hair is ordinarily impermeable in the active condition to aniline blue and acid fuchsin. This is a true case of impermeability of the protoplast, not of unequal distribution of a diffusing substance when equilibrium is reached, for the dye is unable to pass through the protoplast when in contact with either internal or with external surface.

## DIFFUSION OF DYE INJECTED INTO THE CYTOPLASM

When a small injection of either of the dyes named (less than one half the volume of the nucleus) is made in the mesoplasm, it is clearly visible for a moment as a colored region, in contrast to the immediate diffusion of a vacuolar injection. The color promptly fades. All the cytoplasm involved continues streaming without interruption, and no subsequent signs of injury appear. Actually, one cannot here follow the diffusion of the dye into the cytoplasm, but sees instead the disappearance of the color and the absorption of the water. It might be questioned whether this disappearance is due to diffusion from the injection into the cytoplasm or to reduction of the dye. To answer the question, larger injections were made with a two percent solution. From these, the color may actually be seen to spread into the cytoplasm. Larger injections are usually followed after an interval by cessation of streaming, but the cytoplasm into which the dye diffuses is, at the time, in the living, normal condition. Later effects may be due to the action of the dye on the cytoplasm after diffusion.

It would be difficult to reconcile these results with a conception of the entire protoplast as the osmotic membrane of the cell.

## INJECTION OF BROM CRESOL PURPLE
(0.01 M solution, Na salt)

Brom cresol purple is yellow in the acid and blue in the alkaline range. Injected into the vacuole, it takes on the yellow, acid color, regardless of the form in which injected. It shows some toxicity in the vacuole, and causes the protoplasm to give off balls which are carried along by the motion of the sap. When streaming ceases, the indicator in the vacuole slowly changes toward the alkaline color, presumably as a result of inward diffusion of the external medium. The dying protoplasm takes up the dye in the yellow, acid form.

When the dye is injected into the mesoplasm it instantly takes on the blue, basic color, regardless of the form in which injected, and it is in this form that it diffuses into the streaming cytoplasm. A violent, injurious injection takes on the acid color. If a pipette is suddenly withdrawn from a region showing the blue color, the cytoplasm changes to yellow as it escapes explosively.

Since only cytoplasm which has undergone marked injury gives the yellow color to the dye when it is injected into the cytoplasm, and since the yellow form is the only form in which the cytoplasm takes up the indicator which is injected into the vacuole, we have further confirmation of the impermeability of the vacuolar membrane while in the normal state.

## PRESENCE OF FREE ELECTROLYTES IN THE CYTOPLASM

The yellow color of the dye in the vacuole indicates the presence of free hydrogen ions, and the blue color in the cytoplasm must indicate either that the cytoplasm contributes hydroxyl ions or takes up or neutralizes hydrogen ions. Since the color of even a large cytoplasmic injection changes immediately to the alkaline, and the color change takes place before the dye spreads into the cytoplasm, the substance causing the change in reaction cannot be colloidally bound, but must be free to diffuse from the cytoplasm into the injected solution. How can we reconcile the presence of freely diffusible hydroxyl ions, or other ions capable of combining with hydrogen ions, in the cytoplasm with the presence of free hydrogen ions in the vacuolar sap, unless a differentially permeable membrane intervenes? Let us assume for a moment that no membrane is present, but that instead the non-diffusible proteins of the cytoplasm establish a *Donnan equilibrium* with electrolytes in the surrounding solutions. Then the excess of hydrogen ions in the cell sap over that in the cytoplasm indicates that the proteins supply colloidal cations. The cytoplasm should take up hydrogen ions only from solutions more acid than the vacuolar sap. On the contrary, the indicator solution is less acid than the sap, rendering such an explanation of the change in reaction inadequate. Similarly, if we assume that the change in reaction of the indicator is due to adsorption of the hydrogen ions rather than to the diffusion into it of some substance reacting with hydrogen ions, it is difficult to see why the cytoplasm, already in contact with the acid vacuolar sap, should take up hydrogen ions from the slightly acid indicator solution.

## THE EXISTENCE OF DIFFERENTIALLY PERMEABLE PLASMA MEMBRANES

Differential permeability of the vacuolar membrane seems the most reasonable explanation available of the difference in the behavior of the cytoplasm towards the vacuolar sap and towards solutions injected directly into it. The membrane appears to be differentially permeable both to the injected dye and to the electrolytes normally present in the cytoplasm.

## ANOMALOUS BEHAVIOR OF CERTAIN INJECTIONS

Possible causes of anomalous behavior of cytoplasmic injections occasionally seen (Kite, 1913a, and work on *Griffithsia*, this paper) can be determined, thanks to the clearness with which details of injections may be followed in the root hair.

It is worthy of note that it is almost invariably *large* injections which fail to diffuse into the cytoplasm. This is just the reverse of what one would expect if the permeability of the cytoplasm to injected dyes were a result of injury. A large injection will occasionally be sharply delimited from the cytoplasm, the injected drop will tend to become spherical, then suddenly the color will grow more intense, without an accompanying decrease in volume, which would explain the change in intensity by concentration of the solution. Careful focusing shows that the dye has been adsorbed by the (presumably) coagulated surface layer at the dye-cytoplasm interface. A second injection will not pass through such a coagulated film until it is broken, indicating that a coagulated region may be an effective barrier to diffusion. Chambers (1917) suggested formation of a "coagulation film" in explanation of Kite's (1913a) account of "vacuoles" from which the dye does not diffuse as a result of injection in animal cells. The film just described seems to tally with Chambers' suggestion and also with Kite's statement that in some cases the dye is taken up by the wall of the vacuole.

Occasionally one small mass of injured cytoplasm will take up all the dye from the surrounding region, giving a small, intensely colored spot. Such a densely stained coagulum may be what Kite saw (1913a) when he stated that the water may diffuse out from the vacuole formed by injection (starfish eggs) leaving the "precipitated" dye behind.

## EPIDERMAL CELLS OF ONION

The onion material described in the earlier part of the work was used to supplement the work on *Trianea*. It is more difficult to secure a mesoplasmic injection in these cells, for the layer of protoplasm lining the walls is thin, and the details of an injection cannot be followed so easily, but the active streaming makes it possible to check, at all times, on the condition of the injected material.

The method used was varied according to the location of injection desired. Vacuolar injections can easily be secured with the cells mounted in tap water or in an isotonic sugar solution. In plasmolyzing solution the pipette invaginates rather than pierces the protoplast, and the ejected fluid enters neither cytoplasm nor vacuole. In isotonic solutions, or solutions slightly more or less concentrated, it is possible to pierce the wall and plasmalemma without puncturing the tonoplast, and inject into the mesoplasm, with a fair degree of regularity. The greater part of the work was done with the cells in an eight percent solution of sucrose.

## FAILURE OF DYES TO PENETRATE THE PROTOPLAST ON IMMERSION AND VACUOLAR INJECTION

In addition to the dyes used in the work on *Trianea*, phenol red, a hydrogen ion indicator which covers a more alkaline range than that of brom cresol purple, was employed. In order that any effect of sucrose on permeability might affect all methods of application of the dye alike, the solutions for immersion were made up in eight percent sucrose, containing approximately one percent of aniline blue and of acid fuchsin, and 0.005 molar concentration of the indicators. Neither aniline blue nor acid fuchsin visibly penetrated living cells after 28 hours immersion. Neither of the dyes appeared to be toxic. Twenty-four hours in brom cresol purple failed to bring about penetration of living cells. Phenol red occasionally penetrates about one cell out of one hundred, but prolonged immersion produces no increase in the number penetrated, and twenty-eight hours of immersion may produce no penetration whatever.

Acid fuchsin, aniline blue, and phenol red in no case escape from the vacuole when injected before visible abnormalities appear in the cytoplasm, and generally are retained for a while after the death of the cell. Seven hours was the longest observation for acid fuchsin, four hours and forty-five minutes for aniline blue, and ninety-six hours for phenol

red. The greater number of injected cells were only watched for an hour, since it would seem that retention of the dye in the vacuole for an hour spells relative impermeability of the tonoplast when contrasted with immediate diffusion on injection into the mesoplasm.

The toxicity of brom cresol purple prevents the use of this method of testing the permeability of the vacuolar membrane. No visible penetration of the cytoplasm can be seen, however, until the dying protoplasm takes up the yellow, acid form of the dye.

The results of immersion and vacuolar injection indicate that the surface of the protoplast is impermeable to the four dyes whether they are brought into contact with the external or with the internal surface.

## DIFFUSION OF MESOPLASMIC INJECTIONS

Acid fuchsin, injected into the mesoplasm, diffuses almost immediately, without interruption of streaming. The color can be seen to spread in the peripheral cytoplasmic layer. The color leaves the injection more rapidly than the water is taken up by the cytoplasm. The injection usually forms a "blister" in the thin cytoplasmic layer which projects into the vacuole, and this protrusion may persist for a minute or two after the color has left it. The outline of the injection fronting on the mesoplasm is irregular, and becomes more so as the water is taken up, but the appearance presented is definitely indicative that the water is taken up by the cytoplasm; the cytoplasm does not mix with the water. Slight, temporary swelling of the cytoplasm near the injection may follow the absorption of the water.

Did the entire thickness of the cytoplasm constitute the osmotic membrane of the cell, it would be wholly out of order for diffusion of dye to thus precede diffusion of solvent, when solvent alone penetrates from the external milieu.

While the thinness of the cytoplasmic layer increases the difficulty of injection, it also serves to demonstrate more forcibly than in the case of *Trianea* that the mesoplasm is more readily permeable than the tonoplast. In the case of *Trianea*, most injections were made into a mass of cytoplasm through which the dye could spread in all directions. Here, even when the tip of the pipette projects into the vacuole, covered only by a thin film, the injected dye spreads back through this layer into the layer of cytoplasm against the wall. Were the permeability of the tonoplast as great as that of the mesoplasm, this would mean that the dye was diffusing along the path of greatest resistance.

When the medium is slightly hypertonic, so that the pipette fails to pierce the flaccid protoplast, the fluid ejected flows back along the pipette into the external solution, no matter how violently it is ejected. If injury to the cytoplasm were the cause of the permeability shown by diffusion of mesoplasmic injections, one would expect such a violent injection to diffuse into the cytoplasm rather than the small mesoplasmic injection in which no such force is employed.

Aniline blue diffuses in the same manner as acid fuchsin, but is markedly toxic. Coagulation of the injected region results, and this region will not resume streaming, though normal motion may continue in the rest of the protoplast for some time.

## THE DIFFERENTIAL PERMEABILITY OF PLASMA MEMBRANES INDICATED BY INJECTIONS OF INDICATOR DYES

(0.01 M solution, Na salt)

Phenol red in 0.01 M solution has lower coloring power than the other dyes used, hence the injection fades more rapidly as the dye diffuses than does one of acid fuchsin, and the spread of the dye in the cytoplasm cannot be followed well in small injections. Streaming may be locally interrupted, but is soon resumed. The dye takes on the yellow, acid color in the vacuole, and the rose colored alkaline form when injected into the mesoplasm, and, as in the case of brom cresol purple and *Trianea*, the color change is immediate, and takes place before the dye spreads in the cytoplasm. In no case in which local streaming was resumed did phenol red diffuse into the cytoplasm in the yellow form; change to yellow was an indication of injury preceding cessation of streaming. In one case the dye spread in the orange, intermediate form into cytoplasm which showed irregular streaming but swelling followed and streaming soon ceased. Since phenol red covers a more alkaline range than brom cresol purple, it will indicate slighter changes in reaction on injury.

Brom cresol purple is lemon yellow when injected into the vacuole, while a mesoplasmic injection, even when blistering out into the vacuole, immediately takes on the blue color. When injected into the mesoplasm near the nucleus, that body takes up the dye in the alkaline form. When the dye is injected into the vacuole, on the other hand, the nucleus remains uncolored until the dying cytoplasm and nucleus both take up the yellow color. The nucleus may apparently take up the dye from a mesoplasmic injection without injury, for one cell in which the nucleus was colored remained normal for an hour after the injection.

Attempts to contrast the effects of vacuolar and mesoplasmic injection further are not quite safe, for the concentration of the diffusing dye will, of course, vary in the two cases, but it is of interest that brom cresol purple unfailingly produces different cytoplasmic pictures when injected into the vacuole and into the mesoplasm. When it is injected into the vacuole, the mesoplasm of the transvacuolar strands rounds into balls on the tonoplastic portion. Usually Brownian movement of the cytoplasmic granules also results. Vacuolar injections are fatal unless the puncture of the protoplast at the entry point of the pipette enlarges, as described above for puncture by a needle, and the dye is allowed to escape from the vacuole, when the protoplast will survive. When the dye is injected into the cytoplasm, an appearance suggesting alveolation of the mesoplasm, a lace-like pattern, is produced in a large part of the cell, and, over this area, all movement of granules is temporarily suspended. Movement is resumed, the "alveoli" elongate and become irregular in form, and the granules enter into streaming motion. This recovery begins at the region farthest from the center of the injection and proceeds centripetally until all portions of the injected region show normal streaming. There is no balling of strands and no Brownian movedment. So consistently do these two pictures result that it is difficult to avoid the suspicion that the first represents an action of brom cresol purple on the tonoplast alone and the second on the mesoplasm.

The coloring power of brom cresol purple in the basic form is so great that the spread of the dye through the cytoplasm can be traced for half the length of the protoplast or more. The alveolation described usually proceeds slightly farther than the visible diffusion of dye. The question arises, is the alveolation a specific effect of the diffusing dye, or is it indicative of injury which renders the cytoplasm permeable? That the first is the case is indicated by the fact that phenol red, acid fuchsin, aniline blue, eosin, indigo carmine, and isotonic sugar solution can all be injected into and taken up by the mesoplasm without producing this picture. Injection of brom phenol blue gives a similar picture.

The immediate change in reaction of phenol red and brom cresol purple when injected into the mesoplasm, the fact that brom cresol purple can be seen to diffuse half the length of the cell from a small injection, through a thin peripheral layer of cytoplasm, are alike inexplicable unless we picture the mesoplasm as separated from the vacuole by a structure which prevents the passage of substances to which the mesoplasm is freely permeable.

## GRIFFITHSIA

*Griffithsia bornetiana* is composed of branching chains of coenocytic cells which reach a maximum length of 3.2 mm. and breadth of 0.65 mm. The wall, although thick, is soft and is easily pierced with the micropipette. The thin lining layer of protoplasm forms thick pads against the cross walls. By inserting a pipette parallel to the cross wall, it is fairly easy to make a injection into this mass of cytoplasm. The cytoplasm contains numerous nuclei, plastids, some starch, and small protein granules. In the young cell the plastids are closely packed, and polygonal in outline, giving a mosaic pattern. In the older cells the plastids are elongated, oval or irregular in outline, and occur in rows, forming a network. When the cytoplasm is injured, the plastids round up so rapidly that one can follow the change in form, the nuclei swell and become clearly visible, and the protein granules enter into Brownian movement. The change in the plastids and the movement of the granules are the most immediate and striking effects of injury, and may precede loss of color by the plastids by an hour or more, or may occur as reversible injury effects. These changes were used as an index of injury to the cytoplasm.

## FAILURE OF DYES TO PENETRATE ON IMMERSION

Branches of *Griffithsia* were immersed in a saturated solution of eosin in sea water, in 1 gm. of aniline blue to 100 cc. of sea water, and in a one percent solution of methylene blue in sea water, and examined for penetration of the dye after removal to uncolored sea water. The cells were torn open with a microneedle to detect color in the vacuolar sap. Methylene blue gave a faint color to the sap after four hours immersion. Eight hours in the other dyes gave no color to sap or cytoplasm of living cells. Methylene blue was used, as well as eosin and aniline blue, to see if results of cytoplasmic injection showed differences corresponding to results of immersion.

## DIFFUSION OF DYES INJECTED INTO THE CYTOPLASM

The solutions of aniline and methylene blue in sea water were used for cytoplasmic injection. The sea water solution of eosin is too faintly colored to be useful. One percent solutions of all three dyes in distilled water were also used.

Material was kept in hanging drops of sea water, after injection with each of the five solutions, to determine whether injury which was not detectable at the time would make itself known later. One cell, into the cytoplasm of which aniline blue in distilled water had been injected, was in good condition over forty-five hours after the injection. The longest observations for cells injected with other dyes were: eosin, over forty-one hours; aniline blue in sea water, three hours twenty-five minutes; methylene blue in distilled water, four hours and forty-five minutes; methylene blue in sea water, seventeen hours thirty minutes. The injected cells frequently outlived their uninjected neighbors. Injury effects, unless readily recognizable within an hour, failed to appear altogether.

Small injections, from one to four times the volume of a nucleus, are usually wholly absorbed within two minutes. The time required for absorption does not change perceptibly when the dye is dissolved in sea water. The visible color usually passes out of the injected droplet before all the water is taken up. The injected fluid usually shows a sharp margin only where it fronts on the vacuole. Injections diffuse without swelling of cytoplasm, rounding of plastids, Brownian movement of protein granules, or staining of either nuclei or plastids. Slightly larger injections may cause staining of one or two nuclei (aniline blue) or plastids (methylene blue, eosin). Methylene blue shows no perceptible difference from the two acid dyes in behavior on cytoplasmic injection.

In the case of injections so large as to be injurious, the visible injury changes in the cytoplasm usually follow, rather than precede, the diffusion of the dye. Occasionally a "vacuole" with a coagulated, stained, relatively impermeable wall is produced, as described for *Trianea*. It is to be noted that it is only from the large injections that the dye fails to diffuse into the cytoplasm; injury inhibits, rather than facilitates, diffusion.

The plastids make it difficult to determine with finality whether dyes injected into the vacuole might be taken up by the cytoplasm. Eosin is occasionally taken up, but always as the cytoplasm begins to show signs of injury.

The details of injection are less easily followed than in *Trianaea* and onion cells, but it is of interest that all results obtained confirm, for this representative of a wholly different group of plants, those described for the cells above.

## CONCLUSIONS

1. *The differential permeability of the protoplast towards water and dyes is a property of the surface layers rather than the mesoplasm.*

In order to interpret results obtained in any other manner than this, it would be necessary to assume, first, that mechanical injury produces abnormal permeability in the mesoplasm but does not affect the permeability of the two membranes, and second, that the mesoplasm can undergo injury which radically alters its permeability without showing any sign of injury in its appearance or behavior.

2. *The surface layers are also differentially permeable towards freely diffusible electrolytes normally present in cell sap, cytoplasm, and external solution.*

This is shown by instantaneous color changes of injected hydrogen ion indicators in mesoplasm and vacuole, which cannot be explained as results of adsorption or a *Donnan equilibrium.*

3. *The presence of differentially permeable membranes at the surfaces of the readily permeable mesoplasm makes of the vacuolate plant cell a complex system, the behavior of which rests on osmotic as well as colloidal phenomena.*

The mesoplasm evidently retards diffusion, even of water, to some extent. The permeability of the protoplast is influenced by the permeability of all layers, though in most cases the permeability of the external and internal membranes is the limiting factor.

The marked effects of osmotically inactive concentrations of acid and alkali on cell volume result from action on the hydrophilic proteins within the membranes. The intervention of the plasma membrane makes the penetrating power of the acid a contributing factor in its effectiveness.

4. *What is the relation between the morphological membranes, the tonoplast and plasmalemma, and the osmotic membranes?*

Remington (1928) measuring the capacity of the plasma membrane in the beet root, calculates its thickness as 100 Å (0.01 $\mu$). If, however, we alter his calculation by assuming, as the work above would indicate, that each cell contributes *two* membranes, the vacuolar as well as the external plasma membrane, the calculated thickness will be 0.005 $\mu$, or if we assume that the dielectric constant of the membrane is greater than ten, the calculated thickness increases in proportion. Consequently it is impossible to decide from this whether the entire thickness of tonoplast and plasmalemma, or only a surface layer, functions as an osmotic mem-

brane. Since such a layer, if present, is inseparable from the morphological membrane, which gives rise to it, is ruptured when that membrane is ruptured, destroyed when it is destroyed, isolated when it is isolated. it may be permissible in any case to speak of the "differentially permeable tonoplast and plasmalemma" just as we speak of a "waterproof epidermis" when only the cutinized outer wall is really waterproof.

*The plasmalemma and tonoplast are physiological, as well as morphological, entities.*

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## SUMMARY

1. Direct evidence for the existence of distinct osmotic membranes in the living plant protoplast has not hitherto been presented. The presence of a vacuole interferes with efforts to answer the question by indirect methods.
2. Epidermal cells of onion, root hairs of *Trianea*, and the coenocytic cells of the alga *Griffithsia* were used as material for microinjection.
3. It was found that acid dyes which do not penetrate the protoplast immersed in them during its life likewise fail to penetrate the protoplast when injected into the vacuole. On the other hand, when injected into the mesoplasm itself, they were able to spread in the protoplast but unable to pass through the tonoplast into the vacuole or through the plasmalemma into the external solution.
4. This evidence for the existence of osmotic membranes at cytoplasm-wall and cytoplasm-sap interfaces is given further support by color changes of injected indicator dyes, explanation of which is possible only if a membrane differentially permeable to electrolytes occurs at each of these interfaces.
5. It seems reasonably certain that local injury to the cytoplasm did not enter into the results obtained.

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